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The role of anti-endothelial cell antibodies in Kawasaki disease – *in vitro* and *in vivo* studies

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SUMMARY

Kawasaki disease (KD) is a systemic vasculitis with cardiac and noncardiac complications. Anti-endothelial cell antibodies (AECA) are found among many patients with KD. The aim of this study was to investigate the pathogenic role of AECA in KD using *in vitro* and *in vivo* experimental models. $F(ab)_2$ fragments of IgG-AECA and IgM-AECA were affinity purified from a patient with active KD. Their endothelial binding and ability to induce a pro-adhesive and a pro-inflammatory phenotype were evaluated *in vitro*. Twenty Balb/C mice were immunized with KD-AECA or with control Ig (N-Ig) to induce AECA in a murine model by the idiotypic manipulation method. Both KD-AECA isotypes bind significantly to human umbilical vein endothelial cell (HUVEC) compared to N-Ig. The *in vitro* activity was demonstrated by the antibodies ability to activate endothelial cells resulting in increased IL-6 secretion, adhesion molecule expression and monocytic cell line (U937) adherence to HUVEC. Five of the mice that received KD-AECA developed murine AECA after 3 months. None of the mice that received N-Ig produced AECA. The murine AECA increased monocyte adhesion to EC *in vitro*, similarly to the AECA used for immunization. Furthermore, all the mice that developed AECA had proteinuria and IgG deposition in the renal mesangium. No histological or immunofluorescence evidence of cardiac vasculitis could be detected. AECA might play a role in the emergence of some of KD manifestations.

Keywords Anti-endothelial cell antibodies (AECA) Kawasaki disease idiotypes autoantibodies vasculitis

INTRODUCTION

Kawasaki disease (KD) is a systemic vasculitis, diagnosed predominantly in early childhood and characterized by the presence of prolonged fever, conjunctivitis, lymph nodes enlargement and rash [1]. Without prompt treatment, such as intravenous immunoglobulin (Ig), cardiovascular complications are common and some patients may also develop noncardiac manifestations including uveitis, pneumonitis, arthritis or nephritis [2–4]. The pathogenesis of KD is still not completely understood. Many studies suggested that abnormal endothelial functions are central for disease development and for late sequels [5,6]. These studies showed that patients with KD have: (1). Increased in-situ expression and elevated serum levels of cytokines, interleukins, adhesion molecules and growth factors reflecting endothelial cell (EC) activation, damage and regeneration [7–12]; (2). Histological and ultrastructural changes characteristic of EC injury [13,14]. Furthermore, the ability of intravenous Ig to block the EC changes may explain their therapeutic effect in KD [15]. However, the cause of the endothelial dysfunction is unclear. Certain clinical and epidemiological features of KD have suggested that allergens [16], infections [17] or toxins [18] may initiate the disease, yet extensive search has failed to clearly identify such an agent [19,20]. Profound disturbances of immunoregulation, exceeding those accompanying most other febrile childhood illness, implicate that a major immune fault is detrimental for KD development. The immune dysfunctions comprise abnormal apoptosis of circulating neutrophils [21] or mononuclear cells [22]; endothelial tissue infiltration by inflammatory cells [23]; the presence of circulating immune complexes and diverse autoantibody production

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*This work is dedicated in the honour of the late Prof Amiram Eldor who perished recently in a plane crash

[24]. However, none of these immune abnormalities was shown to be central in the pathogenesis of KD. Several studies were able to demonstrate the presence of increased anti-endothelial cell antibodies (AECA) titres in 26% to 72% of the patients with KD [25–27]. Correlation between AECA levels and disease activity and the decline in AECA titres after treatment suggest that AECA may be important in the development of autoimmune and vasculitic diseases and in KD [28–30]. Indeed, several *in vitro* studies reported that sera of patients with KD induced activation or damage to EC, although there were conflicting data concerning the ability of AECA to affect resting *vs.* prestimulated cells [25,27,31,32]. However, as some investigators failed to detect significant difference between the frequency of AECA in patients with KD in comparison to children with other febrile diseases [33], there is still some debate about the actual role of AECA in KD development.

In order to assess directly the role of AECA in KD, we employed an experimental model of active immunization previously used to evaluate the pathogenic role of several autoantibodies. This method is based on Jerne's theory that the idiotypic determinant of each autoantibody is complemented by that of another, creating an idiotypic network. This is manifested by the production of anti-idiotypic antibodies that further stimulate the generation of antibodies to the anti-idiotypic antibody [34]. We [35] and others [36–38], have demonstrated that upon immunization of naïve mice with an autoantibody (Ab-1), an anti-autoantibody (Ab-2) is generated, and four to eight months later an anti-anti-autoantibody (Ab-3) may be detected with similar characteristics as Ab-1. Furthermore, the mice develop clinical and laboratory manifestations typically associated with the human disease from which the inducing autoantibody was obtained [39]. This model proved the pathological role that AECA, anti-neutrophil cytoplasm antibodies (ANCA) and antiphospholipid antibodies (APLA) have in Wegener's granulomatosis (WG) and systemic lupus erythematosus (SLE), respectively [40–42].

The present study provides evidence that AECA derived from a patient with KD can induce the production of mouse AECA followed by clinical and histological abnormalities similar to those observed in KD.

MATERIALS AND METHODS

Immunoglobulin purification and preparation

Serum was obtained from a 2-year-old patient with KD, prior to any treatment, that had a high titre of Igs that bound EC. The patient suffered from 5 days of fever and fulfilled all the criteria for the diagnosis of KD according to the American Heart Association [43], with no evidence of cardiac or renal involvement. He was treated with intravenous Ig and did not suffer from any cardiac or vascular sequels from his disease. Anti-cardiolipin (aCL), anti-dsDNA, anti-ssDNA or ANCA were not detected in his serum. IgG and IgM were purified from the sera on Protein A column (Pharmacia, Upsala, Sweden) and anti-human IgM (Sigma Chemical Co. St. Louis MO. USA), respectively, as described previously [44]. KD-AECA (IgG and IgM) were further purified from the Igs by incubation on confluent monolayers of human umbilical vein endothelial cell (HUVEC). The AECA were then eluted by glycine HCL (0·2 M, pH 2·5), neutralized with Tris buffer and concentrated as previously described [45]. $F(ab)_2$ fragments were prepared from the Igs by pepsin digestion (2% w/w, Sigma Chemical Co.) as described previously [46]. We used two negative controls: pooled Igs from 10 healthy controls (N-Ig) and $F(ab)_2$ fragments of IgG from a patient with Behcet's disease (Behcet's-IgG) that bound significantly less to HUVEC and did not activate HUVEC. $F(ab)_2$ AECA from a patient with Takayasu arteritis (Takayasu-AECA) was used as a positive control [46].

AECA detection

$F(ab)_2$ AECA binding to EC was determined by cyto-ELISA utilizing nonfixed EC, as previously described [46]. Different sources of EC were used: HUVEC [47]; SV-40 immortalized microvascular bone marrow endothelial cells (TrHBMEC) kindly provided by Dr S. Rafii, Weill Medical College of Cornell University, New York, NY USA [48]. To obviate the binding of heterophile antibodies, the preparation was supplemented with 10% fetal-calf-serum [49]. After appropriate washings, affinity purified (a.p.) KD-AECA or N-Ig were added to EC in triplicates, and the binding to EC was evaluated as previously described [46]. The specificity of the Igs binding to HUVEC was also demonstrated by competitive assay. Briefly, cell membranes from washed confluent cultures of HUVEC or TrHBMEC were prepared as previously described [46]. Cells were harvested by mechanical scraping, lysed by freeze thawing three times in PBS containing an enzyme inhibitor EDTA 0·02 M, benzamidine HCL 0·01 M and Trasylol 70 μg/ml. The lysed cell membranes were harvested by centrifugation at 10 000 g for 30 min and the supernant was retained as the cytosolic fraction which was concentrated by ultrafiltration. The pelleted membranes were resuspended in inhibition medium and sonicated four times for 10 s before being centrifugated at 15000 g for 30 min, resuspended in inhibition medium and finally recentrifugated at 4500 g for 15 min to remove cytosolic contamination from the final pelleted preparation. The concentration of KD-AECA producing 50% of the maximal binding to HUVEC-coated plates was determined. Increasing concentration of the HUVEC or TrHBMEC membranes were preincubated with the Igs for 8 h, and placed overnight on HUVEC. The assay was continued as in the AECA cyto-ELISA and the percentage of inhibition was calculated as follows:

$$\% \text{ inhibition} = [\text{OD control} - \text{OD with inhibitor}]/\text{OD control} \times 100.$$

Activation of EC by KD-AECA

The *in vitro* effect of the a.p. $F(ab)_2$ fragments of KD-AECA was assessed by the ability to increase IL-6 secretion, adhesion molecule expression and monocyte adhesion. IL-6 secretion was measured following 4 h of incubation of 10 μg (100 μg/ml) Ig with HUVEC (Immulite IL-6, Diagnostic Products Corporation Calif. USA). E-selectin, ICAM-1 and VCAM-1 expression were measured as previously described [46]. Briefly, HUVEC cells were stimulated overnight with different concentrations of the Igs, treated with PBS-0·2% Triton X-100 and incubated with 1 μg/ml of biotinylated mouse monoclonal antibodies against human E-selectin, ICAM-1 and VCAM-1 (PharMingen, Torreyana Road, San Diego, CA, USA).

The ability of 2×10^4 [^{3}H]thymidine labelled monocytes (U937) to adhere to HUVEC was evaluated as previously described [46]. Prior to monocyte addition, the HUVEC were exposed to increasing Ig concentrations for 12 h (determined by kinetic studies to produce maximal adhesion). The amount of thymidine released from the adherent cells lysed with formic acid was quantified. The results are expressed as percent of added

U937 monocytes that adhered, and are presented as the mean ± SD of triplicate experiments.

Immunization of mice with KD-AECA

Ten μg of Igs derived from the patient (a.p. KD-AECA) or from healthy human controls (N-Ig) dissolved in 100 μl Freund's complete adjuvant were injected into the footpads of 10 healthy BALB/c female mice, 8–12 week old (Tel Aviv University Repository, Tel Aviv, Israel). Four weeks later, the mice were subjected to an identical amount of the appropriate Ig dissolved in incomplete Freund's adjuvant [40]. The study and animal care were approved by the ethical review board, Sheba Medical Centre, Tel-Hashomer, Israel.

Characterization of mouse induced AECA

Every 2–4 weeks the presence of murine anti-human AECA (Ab-2) and mouse AECA (Ab-3) were evaluated. Murine anti-human AECA (Ab-2) were detected by ELISA performed on plates coated with AECA or N-Ig. Briefly, polystyrene plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μg/ml of the $F(ab)_2$ fragments of AECA (IgG) or N-Ig in $NaHCO_3$, pH 9·5. After extensive washes, increasing concentrations of Ig purified from mice immunized with AECA or N-Ig were added for 1 h at RT, and detected with alkaline-phosphatase conjugated goat-anti-mouse IgG (Sigma Chemical Co.) and appropriate substrate.

Mouse AECA (Ab-3) was purified as described for human AECA. Binding of mice AECA to HUVEC and transformed mice endothelial heart cell line H5V (kindly provided by A. Mantovani, Institute Mario Negri, Milan, Italy and maintained in 10% FCS) was assessed by cyto-ELISA and detected with alkaline-phosphatase conjugated goat-anti-mouse IgG (Sigma Chemical Co.) as previously described [50]. The ability of HUVEC membranes to inhibit the binding of mouse AECA to H5V and the murine AECA effect on monocyte adhesion to HUVEC were measured as described above.

Clinical manifestations in immunized mice

Proteinuria was measured by combistix kit (Ames, Elkhart, IN) and haematuria was evaluated by microscopic visualization. Six months after the first injection, the mice were sacrificed and the heart, aorta, lungs, kidney and liver were examined histologically following haematoxylin-eosin staining. Immunofluorescent studies were performed with FITC-conjugated goat-anti-mouse IgG Fc-specific antibodies (Sigma), as previously described [40]. Three specimens of each organ were examined independently by two of the authors (AA and KJ).

Statistical analysis

Statistical analysis was done by employing the ANOVA statistics software Inc, and a result was considered as statistically significant if $P < 0{\cdot}05$ or differed more than three standard deviations from the control group average. Significance of correlation was calculated with Student's *T*-test or Spearmans's test.

RESULTS

Characterization of KD-AECA

In order to characterize the biological activity of AECA derived from the patient with KD we analysed its ability to bind macrovascular and microvascular EC and followed its potential to activate these cells. Concentration-dependent binding (at 6·25–100 μg/ml) of KD-AECA (IgG and IgM) to HUVEC but not to TrHBMEC was found, and representative data are reported in Table 1. Furthermore, the specific binding of the patient's Ig to HUVEC was demonstrated also by the effect of preincubation with HUVEC membranes (Fig. 1). Incubation of KD- AECA and Takayasu-AECA (at the concentration of 50% of maximal binding [25 μg/ml]) with increasing concentrations of HUVEC membranes caused significant inhibition of binding compared to the effect of TrHBMEC membranes ($P < 0{\cdot}05$). Incubation with either EC membranes had np effect on the binding of pooled Ig from 10 normal controls or Behcet's Ig (data not shown).

Table 1. Binding of AECA from a patient with Kawasaki disease to endothelial cells

	HUVEC	TrHBMEC
IgG-AECA	1·478 ± 0·234*	0·197 ± 0·098
IgM-AECA	1·167 ± 0·252*	0·152 ± 0·078
Takayasu-IgG	1·504 ± 0·244*	0·142 ± 0·078
Behcet's IgG	0·903 ± 0·102**	0·143 ± 0·064
N-IgG	0·114 ± 0·024	0·104 ± 0·046
N-IgM	0·128 ± 0·156	0·132 ± 0·052

Binding of 100 μg/ml of a.p. $F(Ab)_2$ fragments to human umbilical vein endothelial cells (HUVEC) and SV-40 immortalized microvascular bone marrow endothelial cells (TrHBMEC). Ig are from a patient with Kawasaki disease (IgG-AECA and IgMAECA), polyclonal IgG from a patient with Takayasu arteritis, Behcet's disease and pooled IgG from 10 controls (N-IgG and N-IgM).

Values are the mean ± SD optical density at 405 nm of triplicate measurements from 3 separate experiments. *$P < 0{\cdot}001$ compared to N-Ig, **$P < 0{\cdot}01$ compared to N-Ig.

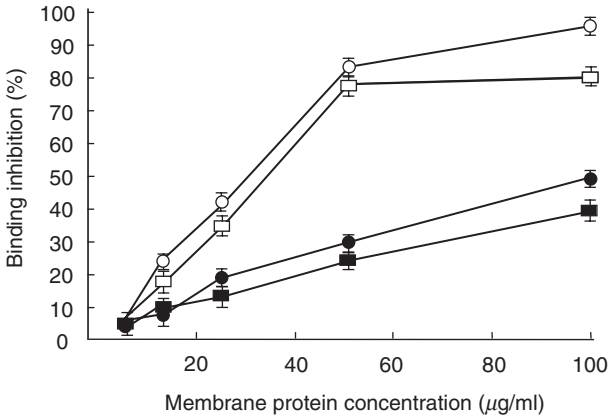

Fig. 1. Binding inhibition of AECA binding to HUVEC. Anti-endothelial cell antibodies from a patient with Kawasaki disease (KD-AECA) at 50% of maximal binding (25 μg/ml) to human umbilical vein endothelial cells (HUVEC) were incubated with increasing concentrations of membrane protein preparations from HUVEC or microvascular EC (human bone marrow EC immortilized by SV-40 [TrHBMEC]). ● Takayasu Ig + TrHBMEC; ○ Takayasu Ig +HUVEC; ■ KD-AECA + TrHBMEC; □ KD-AECA +HUVEC; Takayasu Ig, IgG from a patient with Takayasu arteritis. Values represents the mean ± SD of triplicate measurements from 3 separate experiments.

Table 2. Adhesion molecule expression following AECA activation of endothelial cells

	E-selectin	ICAM-1	VCAM-1
IgG KD-AECA	0·586 ± 0·102*	0·624 ± 0·098*	0·544 ± 0·108*
IgM KD- AECA	0·511 ± 0·088*	0·562 ± 0·152*	0·598 ± 0·088*
Takayasu-IgG	0·722 ± 0·134*	0·844 ± 0·122*	0·698 ± 0·136*
Behcet's IgG	0·156 ± 0·066	0·078 ± 0·036	0·084 ± 0·044
N-IgG	0·098 ± 0·046	0·080 ± 0·046	0·088 ± 0·044
N-IgM	0·086 ± 0·054	0·092 ± 0·038	0·074 ± 0·032

Adhesion molecule expression following exposure to 25 μg/ml of AECA from a patients with KD (IgG and IgM), IgG from patients with Takayasu arteritis and Behcet's disease and control (IgG and IgM). Values are the mean ± SD optical density at 405 nm of triplicate measurements from 3 separate experiments. $P < 0{\cdot}001$ compared to N-Ig.

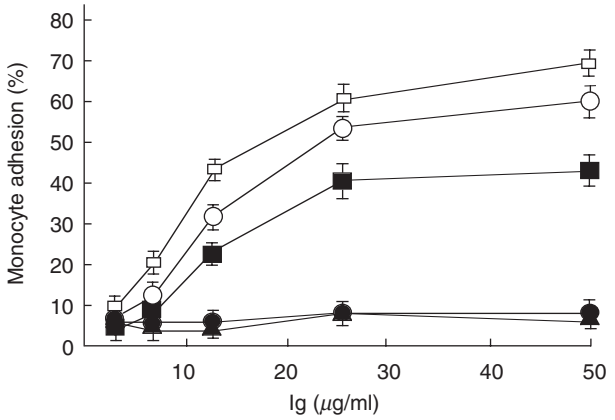

Fig. 2. Monocyte adhesion to endothelial cell activated by KD-AECA. Adherence of [^{3}H]-thymidine labelled U937 monocytes to human umbilical vein endothelial cells stimulated with increasing concentrations of AECA (○, IgG or ■ IgM) from a patient with Kawasaki disease (KD) or control (▲ N-IgG; ● N-IgM). The results are expressed as percent of added U937 cells that adhered, and are presented as the mean ± SD of triplicate measurements from 3 separate experiments. □ Takayasu-Ig.

The ability to activate EC was determined by IL-6 secretion, adhesion molecule expression and monocyte adhesion. Similar to the effect of the IgG from Takayasu arteritis, AECA-IgG and IgM increased significantly ($P < 0{\cdot}001$) the IL-6 secretion from HUVEC (both isotypes >3000 ng/ml), compared to N-IgG (47 ng/ml), N-IgM (103 ng/ml) or Behcet's-IgG (52 ng/ml). AECA induced a concentration-dependent increase in E-selectin, ICAM-1 and VCAM-1 expression and representative results at 25 μg/ml are displayed in Table 2. However, initial VCAM-1 expression upon KD-AECA stimulation was observed only at 12·5 μg/ml compared to 3·125 μg/ml for E-selectin and ICAM-1 expression.

Incubation with AECA increased significantly the adhesion of U937 monocytes to the HUVEC compared to that of N-Ig (Fig. 2).

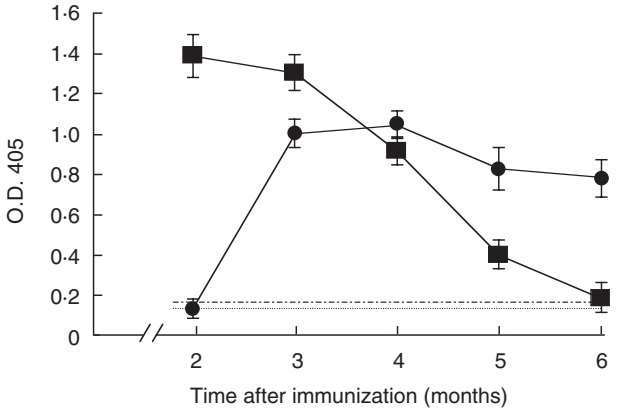

Fig. 3. Murine anti-human AECA and murine AECA. Murine anti-human AECA (Ab-2; ■) and murine AECA (Ab-3; ●) in the mice that developed AECA following immunization with affinity purified Ig from a patient with KD-AECA (KD-AECA). Values represent the mean ± SD from 3 separate experiments. ····· Binding of Ab-2 to control-Ig (mean O.D. ± 3 SD). –·–·– Binding of Ig from mice immunized with control-Ig to murine H5V endothelial cells (mean O.D. ± 3 SD).

Induction of murine AECA upon immunization with KD-AECA

After demonstrating the *in vitro* effect of KD-AECA, it was injected into BALB/c mice. Two months after immunization, significantly higher titres of murine anti-human AECA (Ab-2) reacting with the patient's $F(ab)_2$ AECA were detected in all the mice immunized with KD-AECA (Fig. 3). Three months after the first injection, significantly higher titres of murine AECA were evident in 5 of the mice injected with KD-AECA, compared to the levels in mice subjected to N-Ig. The mouse AECA titres peaked one month later and did not decline significantly until the mice were sacrificed.

Characteristics of the murine AECA

Increasing concentrations (3·125–50 μg/ml) of murine AECA (Ab-3) showed high binding to mouse H5V EC and HUVEC, compared to Ig from mice immunized with N-Ig (mean ± SD O.D. at 405 nm $1{\cdot}103 \pm 0{\cdot}075$ and $1{\cdot}045 \pm 0{\cdot}098$ *vs.* $0{\cdot}135 \pm 0{\cdot}030$ and $0{\cdot}108 \pm 0{\cdot}045$, respectively; $P < 0{\cdot}001$). The cross-reactivity between the antigens presented on human EC and murine EC was confirmed by a dose-dependent inhibition of binding of the mouse AECA (at 50% of maximal binding- 12·5 μg/ml) to H5V by increasing concentration of HUVEC membranes (Fig. 4). The murine AECA induced a concentration-dependent adhesion of monocytes to HUVEC (Fig. 5).

Induction of vasculitis after KD-AECA immunization

Proteinuria was noted 4 months after immunization in the urine of all the mice in which murine AECA were found, while none of those injected with the KD-AECA that did not develop AECA or with N-Ig had any abnormal urinary findings. Furthermore, there was also a significant diffuse fluorescent staining in the renal mesangium of the mice that developed murine AECA which was not evident in the kidneys of the mice immunized with N-Ig (Fig. 6). However, haematoxylin-eosin preparation of the aorta, heart, liver, kidney or the lung did not disclose any vasculitis, and no increased immunofluorescence was detected in these tissues.

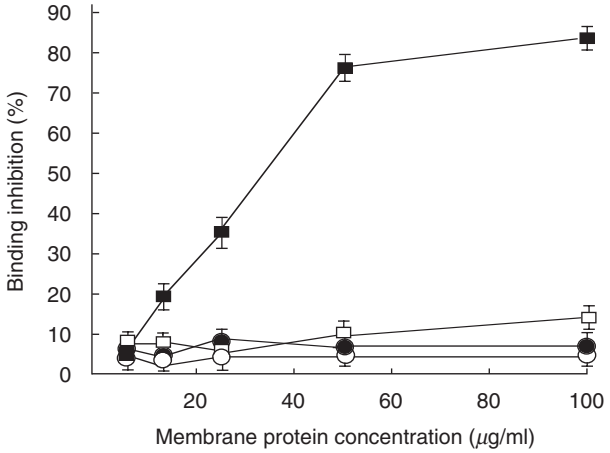

Fig. 4. Binding inhibition of murine AECA to murine endothelial cells. Inhibition of binding of murine AECA (at 50% of maximal binding-12·5 μg/ml) to mouse H5V endothelial cells by increasing concentration of HUVEC membranes, but not by human bone marrow microvascular EC immortilized by SV-40 (TrHBMEC) membranes. Values represent the mean ± SD of triplicate measurements from 3 separate experiments. mAECA, Murine AECA from mice immunized with human AECA; N-Ig, IgG from mice immunized with control human IgG. ■ mAECA + HUVEC, □ mAECA +TrHBMEC, ● N-Ig + HUVEC, ○ N-Ig + TrHBMEC.

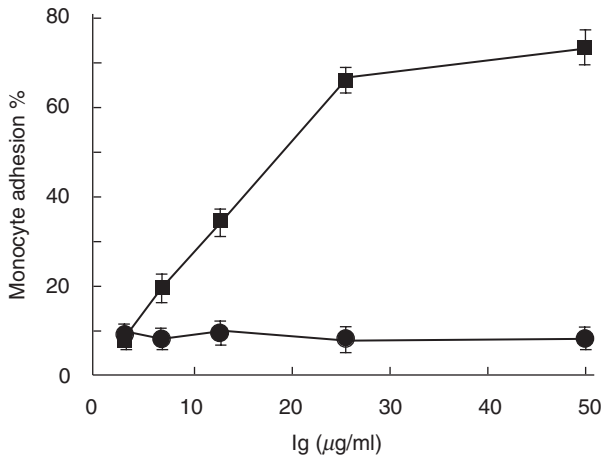

Fig. 5. Monocyte adhesion to human umbilical vein endothelial cells. Adhesion of [^{3}H]thymidine labelled U937 monocytes to human umbilical vein endothelial cells subjected to mouse AECA (mAECA, ■) or Ig from mice immunized control (N-Ig, ●). Each point represents the mean ± SD of triplicate measurements from 3 separate experiments.

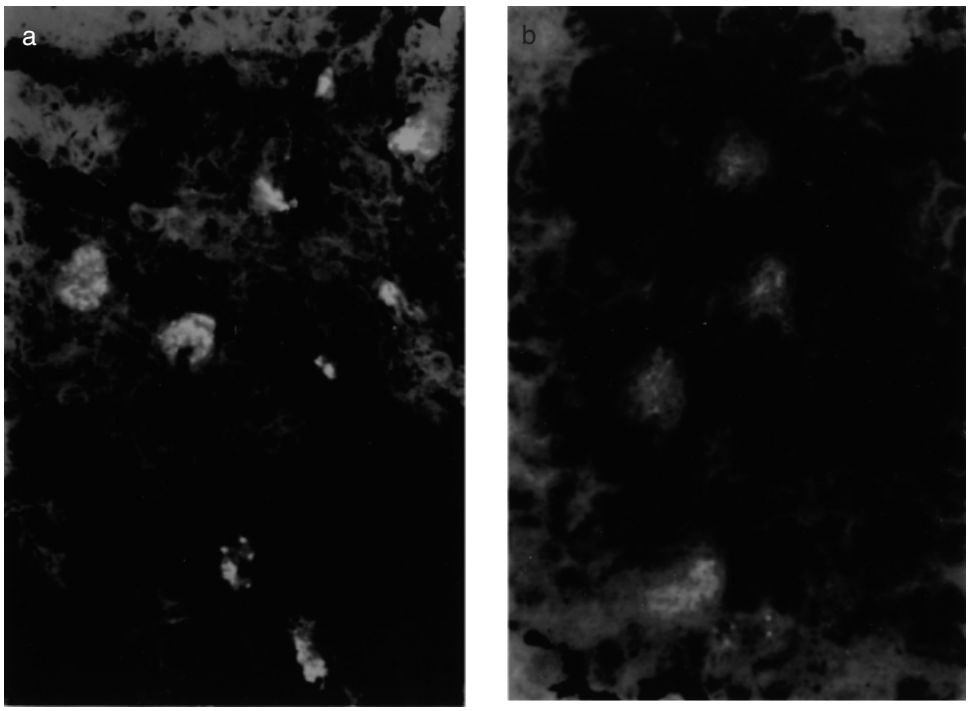

Fig. 6. Immunofluorescent staining of kidney. IgG is detected in the mesangium of mice that developed murine AECA (KD-AECA) (a) and not in those immunized with control Ig (N-Ig) (b). Kidney section (5 μm) stained with FITC-labelled anti-murine IgG (× 1000).

DISCUSSION

AECA are implicated in the pathogenesis of several autoimmune and vasculitic diseases [35]. Some of the *in vitro* effects of AECA derived from patients with KD were investigated previously [25,31], however, the importance of AECA in KD development remains debatable [26,33]. Therefore, we assessed the *in vivo* role of AECA by inducing its formation utilizing the idiotypic manipulation method [39].

In previous experimental models of idiotypic manipulations, autoantibodies with *in vitro* effects were employed for the induction of autoimmunity [40,50,51]. Therefore, prior to mice immunization the ability of a.p. KD-AECA to actually affect EC function *in vitro* was evaluated. Indeed, a.p. KD-AECA increased cytokine secretion, EC adhesion molecule expression and monocyte adhesion to HUVEC, similar to the effect of AECA from patients with other vasculitic or autoimmune diseases [46,52]. In agreement with previous suggestions [9,15,21,27,53,54], our experiments emphasize a possible mechanism that may explain the role of AECA in KD development. AECA may favour the induction of a pro-inflammatory endothelial phenotype, thereby contributing to the inflammatory vasculitic infiltration and eventually to EC destruction.

Interestingly, KD-AECA were able to affect resting HUVEC. Conflicting data has been presented previously on the necessity of EC preactivation. Some reported that AECA lyse-cytokine activated cells [31,32] while other studies demonstrated that KD-AECA influences resting cells as well [25,27]. Our results agree with the latter and with the ability of AECA from other diseases such as Takayasu arteritis, thrombotic thrombocytopenic purpura, and SLE to affect nonactivated EC [45,46,55]. However, we cannot completely role out the possibility that concomitant cytokine stimulation further increases EC susceptibility [27] or that the *in vitro* manipulation induces EC activation, which emphasizes the importance of an *in vivo* model.

Similarly to previous models of idiotypic induction of autoimmune diseases [35,39], the mice immunized with Ab-1 (AECA from the patient with KD) developed anti-AECA antibodies (Ab-2), followed by the appearance of anti-anti-AECA (Ab-3). Murine anti-endothelial antibodies (Ab-3) were detected in only 5 of the 10 mice that developed Ab-2. We were not able to identify any factor predisposing to Ab-3 production including the level of Ab-2 (anti-Id). Discordance between Ab-2 and Ab-3 production was observed in similar animal models [41]. Ab-1 and Ab-2 were polyclonal antibodies each recognizing numerous epitopes, therefore some variability in the properties of the ensuing antibody is expected. However, as expected from 'Jerne's idiotypic network' theory, the murine AECA bound and increased monocyte adhesion to EC similarly to that observed with the primary human AECA (Ab-1).

Murine AECA reached the highest levels 4 months after the immunization, which paralleled the development of proteinuria. Although renal involvement is not a common feature in KD [2,3] it was described and manifested most often as proteinuria [4]. The cause of the proteinuria is not completely clear, however, increased levels of several cytokines are found among patients with KD, including IL-6 [56–58], and the levels of these cytokines are in correlation with disease progress [59]. Increased IL-6 has been implicated in the pathogenesis of the KD renal lesions [60] as well as IgA nephropathy and Henoch-Schonlein purpura [61]. Indeed, the KD-AECA utilized in this study increased HUVEC's IL-6 production. Immune-complexes have also been suggested as a possible mechanism for the nephrotoxicity in KD [4] and they are reported in many patients with KD [24]. We detected IgG in the renal mesangium of the mice that developed AECA, which could represent deposition of immune-complexes in the kidney. Similar mesangial findings have been reported recently in KD [62] as well as in several vasculitides such as Henoch-Schoenlein purpura and immune-complex diseases [63–65]. Thus, the renal damage in KD may be related to AECA mediated EC activation and cytokine secretion as well as to AECA immune-complex deposition.

KD is a heterogeneous disease, which may have different aetiologies and pathogenic pathways. It is therefore not surprising that the presence and frequency of AECA among patients with KD were different in several reports. We did not try to clarify whether AECA is present and pathogenic in all or even in a significant proportion of patients with KD. Rather, we attempted to identify the possible role that AECA may have in KD when it is present. We selected Ig from a patient with KD that had a high titre of both IgG and IgM AECA that adhered to HUVEC, as both AECA isotypes are increased in patients with KD [27]. AECA are a heterogeneous group of antibodies. We established previously that while some of the different AECA have similar ability to activate EC, they may also be associated with different clinical features and induction of different effects in animal models [66]. AECA derived from a patient with WG cause vascular damage of the lung and kidneys while antibodies reacting with EC derived from patients with heparin induced thrombocytopenia are associated with the development of thrombocytopenia in mice. Therefore, we concentrated on the effect of KD-AECA, although certainly AECA from other diseases or even from febrile patients without vasculitis [33], with the appropriate conditions, might cause similar effect as KD-AECA. Some investigators have concentrated on the *in vitro* and *in vivo* pathogenic role of monoclonal AECA [46,52]. In order to evaluate as many antibodies as possible we chose to start our research with a polyvalent preparation, that similar to other reports [67–69], immunoprecipitated a wide molecular weight range of target molecules (data not shown). However, this forced us to concentrate on a single patient's serum, which although had classical clinical manifestations of KD and a high AECA titre might not be representative of all patients with KD and might not contain all the possible repertoire ofAECA-KD.

Previous murine model induced by idiotypic manipulation with AECA from patients with WG exhibited lung and kidney involvement with perivascular lymphocyte infiltrate and IgG deposition at the outer part of the blood vessels in the kidneys. As these organs are affected in patients with WG, the histological and immunofluorescent findings in the WG model are thought to represent the inflammatory vasculitic process [40]. Unlike the WG model, and despite an extensive evaluation for histological abnormalities described in patients with KD [13,70], there was no indication of vasculitis in the mice immunized with a.p. KD-AECA. The BALB/c mice strain used in the current study tends to develop coronary vasculitis after an appropriate stimulation (*Lactobacillus casei* cell wall) with acute and chronic histopathological changes that closely resemble those found in children with KD [54]. However cardiac vasculitis occurs in less than 15% of the children and the incidence is even lower later in the course of the disease [71]. The same may be true regarding the incidence of vasculitis in mice. Furthermore, numerous murine models of human

diseases were not able to completely produce all the clinical and laboratory manifestations. The lack of blood vessel involvement in the current idiotypic manipulation model could also be due to the following: (1) Absence of a target autoantigen on murine EC. Although the target antigens for Ab-1 and Ab-3 were not identified, the ability of murine Ab-3 to react with both HUVEC and murine EC indicates that some common antigens are present on both cell types and cross-reactivity between human and murine EC has been reported previously [50]. The latter is also demonstrated by the ability of the HUVEC to inhibit the binding of murine AECA to murine EC; (2) Lack of concomitant stimulation of EC by cytokines. Increased cytokine production and increased cytokine levels in the sera have been found in KD, which may increase the susceptibility of EC to injury [7,8,72]. We demonstrated that AECA affect EC *in vitro* without other stimuli, however, the magnitude of such effect *in vivo* may be insufficient to cause clinical vasculitis. Thus, we hypothesize that although AECA have a pathologic role in the development of some of the KD features, they do not initiate or are not the main cause of the cardiac vasculitis. Other important factors, such as infections, exposure to superantigens or toxins or stimulation of EC by cytokines may be necessary for disease development, at least in some of the children with KD.

In conclusion, AECA from KD directly affect EC function and may be responsible for the renal abnormalities observed in some of the patients. The data derived from this experimental model suggest that AECA are not the primary cause of the cardiac vasculitis, and the presence of other factors may be necessary for development of clinical manifestations typical of KD.

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